

## Targeting the Shikimate Pathway in the Malaria Parasite *Plasmodium falciparum*

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**The shikimate pathway presents an attractive target for malaria chemotherapy. Three shikimic acid analogs exhibited different effects on *Plasmodium falciparum* growth. (6*R*)-6-fluoro-shikimate and (6*S*)-6-fluoro-shikimate inhibited growth (50% inhibitory concentrations,  $1.5 \times 10^{-5}$  and  $2.7 \times 10^{-4}$  M, respectively), whereas 2-fluoro-shikimate had no effect. *para*-Aminobenzoic acid abrogated the inhibition, demonstrating that the shikimate pathway was specifically targeted.**

Earlier studies with auxotrophic mutants demonstrated that *para*-aminobenzoate (pABA) synthesis is essential in *Plasmodium falciparum* (9). pABA, a key intermediate in folate production, is synthesized via the shikimate pathway. Several enzymes have been detected in *P. falciparum* extracts, and a gene encoding one of these enzymes has been identified (3, 13).

The shikimate pathway, conserved in plants, algae, bacteria, and fungi, has recently been detected in several apicomplexan parasites (13). The phylum Apicomplexa consists of intracellular protozoan parasites, including *Plasmodium*, that cause substantial mortality, morbidity, and economic losses. The shikimate pathway, also termed the aromatic biosynthetic pathway, is a series of seven enzymes that generates the common aromatic precursor chorismate from simple products of carbohydrate metabolism (7, 11). Chorismate is metabolized to pABA, ubiquinone, and the aromatic amino acids. In plants, the shikimate pathway is localized to the chloroplast. This is intriguing, as apicomplexan parasites contain a chloroplast-related organelle that is essential and can be specifically targeted (8, 5, 10, 14). The shikimate pathway may be localized in this organelle or in the cytoplasm, as found in fungi and bacteria.

The absence of the shikimate pathway in mammals presents an excellent target for development of new chemotherapeutic agents. Fluorinated analogs of shikimate have potency against bacteria (2). They interrupt pABA synthesis, analogously to aromatic-deficient auxotrophs of bacteria (2, 16). In this study shikimate analogs were tested for their effect on *P. falciparum*.

Initially, attempts were made to grow *P. falciparum* in medium deficient in aromatic metabolites of the shikimate pathway. The medium was similar to that previously described, lacking pABA and folate (9). In this study the medium additionally lacked phenylalanine, tryptophan, and tyrosine, and a serum-free substitute (Albumax I; GIBCO BRL) replaced the serum. A clonal *P. falciparum* strain (3D7) was found to grow in human erythrocytes at a lower rate ( $\sim 70\%$  of the growth rate in standard medium) when introduced into aromatic-deficient medium. Similar requirements for these components have previously been observed (4). Therefore, exogenous aromatic compounds are unnecessary for growth of asexual blood stages of *P. falciparum*, supporting the role of the shikimate pathway in supplying aromatic compounds.

Three fluorinated analogs of shikimate were tested for their effect on *P. falciparum*. The intermediate shikimate in the pathway is formed from erythrose 4-phosphate and phosphoenolpyruvate by four enzymatic steps (7, 11). The analogs contain a fluorine substitution for a hydrogen in one of three positions (Fig. 1). The two compounds containing a fluorine at the C-6 position (2) are stereoisomers (kindly provided by ZENECA Pharmaceuticals, Alderley Park, Macclesfield, United Kingdom). These compounds have antibacterial activities, but (6*S*)-6-fluoro-shikimic acid (henceforth in this work termed compound A) is 250- to 600-fold more potent than (6*R*)-6-fluoro-shikimic acid (henceforth in this work termed compound B). The third shikimate analog, 2-fluoro-shikimic acid (henceforth in this work termed compound C), was a kind gift from P. A. Bartlett (University of California, Berkeley) (12). Parasites were treated, in triplicate, as previously described (9). Levels of resulting parasitemia were determined microscopically after two cycles of growth with a single change of medium. These results were reproducible in repeated experiments.

The analogs had quite different effects on the growth of *P. falciparum*. The 6-fluoroshikimates inhibited parasite growth in a dose-dependent manner (Fig. 2). Compound B was more potent than compound A (50% inhibitory concentrations of  $1.5 \times 10^{-5}$  and  $2.7 \times 10^{-4}$  M, respectively). In contrast, compound C had little detectable effect on the growth of parasites (data not shown). The sensitivity of *P. falciparum* to compounds A, B, and C is compared to its sensitivity to thiostrepton (Fig. 2), an inhibitor of protein synthesis in the plastid-like organelle (10).

The difference in sensitivity to the compounds suggests a specific mechanism of inhibition. *P. falciparum* is 18-fold more

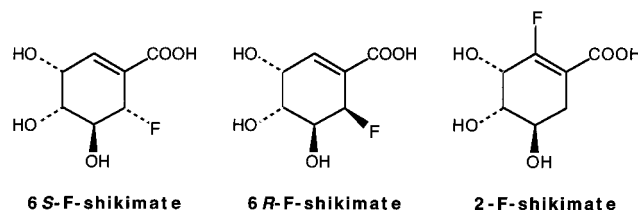


FIG. 1. Structures of fluorinated analogs of shikimic acid: (6*S*)-6-fluoro-shikimic acid (ZM 240401), (6*R*)-6-fluoro-shikimic acid (6 *R*-F-shikimate) (ZM 218463), and 2-fluoro-shikimic acid (2-F-shikimate), referred to as compounds A, B, and C, respectively, in the text. Structures are redrawn from references 2 and 12.

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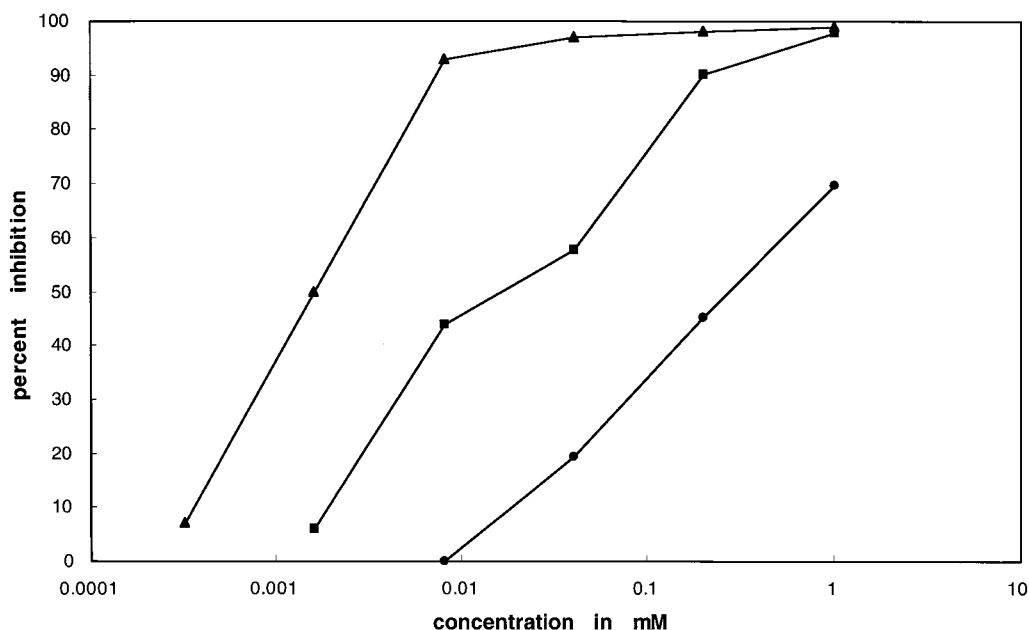


FIG. 2. Effect of fluorinated analogs of shikimic acid on growth of *P. falciparum*. The inhibition in the number of parasites is expressed as a percentage of control cultures. The percent inhibition is plotted against the concentration of compound A (●), compound B (■), and thiostrepton (▲).

sensitive to compound B than compound A and insensitive to compound C at the concentrations tested. This contrasts the 200-fold-greater sensitivity of *Escherichia coli* to the compound A stereoisomer (2). The lack of effect of compound C on parasite growth may be because the C-2 hydrogen is not involved in aromatic biosynthesis.

If the shikimate pathway is being specifically inhibited, then the inhibition should be antagonized by supplementation with exogenous aromatic compounds. As *P. falciparum* was most

sensitive to B, the following studies concentrated on this analog. Cultures were treated in medium containing tryptophan (5 mg/liter), tyrosine (20 mg/liter), phenylalanine (15 mg/liter), *para*-hydroxybenzoate (pHBA) (10 mg/liter), and pABA (10 mg/liter). The increase in parasite number, in triplicate experiments, was monitored after 3 days as explained above. This medium abolished the inhibitor effects (Fig. 3) even at the highest concentration of inhibitor tested (1 mM). Therefore, the inhibition is specific to the shikimate pathway.

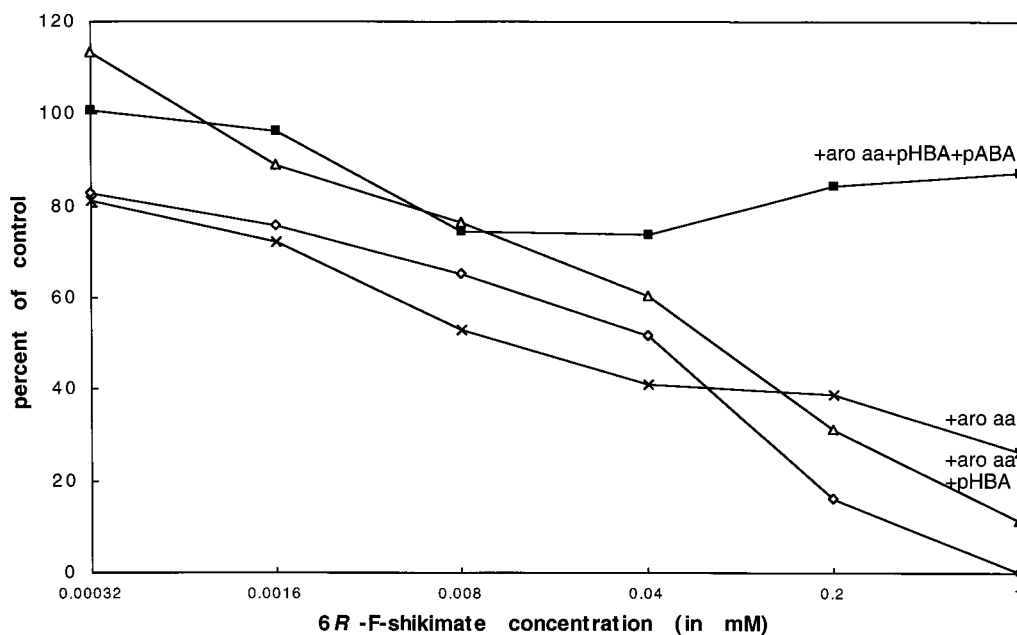


FIG. 3. Antagonism of growth inhibition by (6R)-6-fluoro-shikimic acid. Results represent the growth of *P. falciparum*, calculated as a percent of control, in increasing concentrations of compound B in the medium described in the legend to Fig. 2 (◇) or media containing aromatic amino acids (+aro aa) (×), +aro aa and pHBA (△), and +aro aa, pHBA, and pABA (■). The standard deviation for all points is  $\leq 6.2$ .

As pABA synthesis is essential for parasite growth (9), its requirement in reversing inhibition was examined. Parasites were treated in medium lacking pABA, folate, and pHBA but supplemented with aromatic amino acids (Fig. 3). Parasites were also treated in medium lacking only pABA and folate (Fig. 3). Neither combination abrogated the effect of the inhibitor. Only when the pABA was included in the medium was the inhibition abolished (Fig. 3). Hence pABA is necessary for antagonizing inhibition by compound B. These results concur with observations in bacteria and fungi. In these organisms, compound B exerts its effect on chorismate synthesis (1). In *P. falciparum* this effect is most likely exerted through inhibition of pABA synthesis, as these parasites can salvage amino acids from the host cell (6, 15).

The sensitivity to shikimate analogs suggests that the shikimate pathway is viable for malaria chemotherapy. The 50% inhibitory concentrations of these analogs are below those of some currently used antimalarial drugs (13). Several apicomplexan parasites have recently been found to be sensitive to *N*-(phosphonomethyl) glycine (glyphosate) at concentrations of 1 to 6 mM (13). Glyphosate is a potent and specific inhibitor of the shikimate pathway. As in this study, the inhibition by glyphosate was abrogated by the addition of pABA to the medium. This suggests that glyphosate and compound B exert their effects by a similar mechanism. Therefore, shikimate analogs may act as universal inhibitors of apicomplexan parasites, such as *Toxoplasma gondii* and *Cryptosporidium parvum*, which cause opportunistic infections in patients with AIDS.

Based on the observations that mice were protected by 6-fluoro-shikimate from intraperitoneal bacterial infection (2) and that mice were cleared of *Toxoplasma* by treatment with a glyphosate-pyrimethamine formulation (13), the effectiveness of 6-fluoro-shikimate on malaria treatment awaits testing in rodent models.

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